EFFECTS OF ESTROGENS ON ASTROCYTES EXPOSED TO STRESSORS

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ABSTRACT

Hormone replacement therapy (HRT), used by many women to alleviate menopausal symptoms such as hot flashes and mood swings, is often a combination of hormones such as estrogens, progesterone, and conjugated equine estrogens (CEE), extracted from the urine of pregnant mares. Previous studies have found positive correlations between estradiol and cellular protection, but recent research has concluded CEE provide less protective mechanisms as compared to endogenous hormones. This research sought to compare the effects of estrogen treatments (single and combined estrogen) on viability when astrocytes were induced with stressors (epinephrine, cortisol, and low oxygen concentration). Cultured human astrocytes were treated with 17β-estradiol and equilenin, either alone or in combination. Following estrogen treatments, astrocytes were induced with stressors, and an MTT assay was used to measure cell viability. Estradiol was expected to provide the most protection as a single hormone treatment for all three stressors. Higher concentrations of equilenin either alone or in combination with estradiol yielded significantly lower cell viability following epinephrine and cortisol stressors. There was no viability difference found in astrocytes stressed with low oxygen concentration. The analysis of this research helped to elucidate the relative protective effects of two forms of estrogens. Future research on estrogen binding using primary human astrocytes and neurons would help further understand the neurological effects of estrogens neurologically. The implications of this study suggest HRT could be detrimental to neurological cells, and these negative effects are dose dependent.

INDEX WORDS: Estradiol, astrocytes, equilenin, cell viability, glial cells, neuronal protection, menopause, estrogen effects, Premarin, protective effects of estrogens

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INTRODUCTION

Estrogens

Estrogens mediate many functions in the body and regulate homeostasis. Women who have a reduced estrogen production in the perimenopausal, menopausal, and postmenopausal stages suffer associated symptoms from decreased estrogen production in the body such as fatigue, mood swings, osteoporosis, hot flashes and night sweats (Brunner et al. 2010). Supplemental estrogens and hormones are often prescribed to relieve these symptoms. These combinations frequently include estradiol, estrone (found in higher concentrations of pregnant women), and progesterone. Estradiol includes two common subtypes found in many supplemental estrogens which are 17α-estradiol and 17β-estradiol. Estradiol is produced within the follicle of the female ovaries. It plays a major role in the reproductive organs of women as well as the lining of the fallopian tubes, vagina, cervical glands, endometrium, and maintaining oocytes in the ovaries (Finstad et al. 2009). In premenopausal women, estradiol levels decrease from 15-350 pg/mL to often less than 10 pg/mL in postmenopausal. 17α-estradiol and 17β-estradiol compounds only differ slightly in position of a hydroxyl group and these compounds have similar change during menopause (Rotti et al. 1975). Estrone levels also drop significantly as women age. This drop in estrone can range from 17-200 pg/mL in premenopausal women to 7-40 pg/mL in postmenopausal women (Rotti et al. 1975). Since most hormones maintain homeostasis in the body, fluctuations of estrogens and progesterone such as in menopause often leads to hot flashes, night sweats and mood changes (Kardong 2015; Regidor 2014).

Evidence of Neuroprotection by Endogenous Estrogens

In addition to alleviating menopausal symptoms, the clinical use of estrogen also offers cellular protection when exposed to toxins such as glutamate and β-amyloid protein (Zhao and Brinton 2006). Estradiols and estrone showed significant protection in rat neurons when induced with various stressors (Zhao and Brinton 2006). Estradiols, specifically 17β-estradiol, contribute to the regulation of brain activity, structural proteins synthesis, estrogen receptors, and enzymes (Tozzi et al. 2015). Much research has confirmed a positive correlation between estrogens and memory (Tozzi et al. 2015; Foy et al. 2000).

The protective effects of estradiol were seen in a study conducted by Mosquera et al. (2014). This research tested the neuronal protective effects of estradiols and tamoxifen, an estrogen receptor mixed agonist/antagonist. Rats were induced with a spinal cord injury seven days after their ovaries were removed. Ovariectomy allowed researchers to quantify levels of hormones being tested and to assure accurate results related to the effects of hormones on spinal cord injury recovery. The hormones were administered to the rats orally at a concentration of 3 mg in food pellets. Oxidative stress was then induced. Locomotion was measured at 7, 14, 21, and 28 days post injury. Improved rates of locomotion were seen at days 21 and 28 for the rats receiving tamoxifen. However, locomotion improved in rats receiving estradiol at all four times measured. The conclusion of this study implies that both hormones could potentially be used as a long-term treatment in the recovery from spinal cord injury and as a general neurological protective agent.

Traumatic brain injuries result in permanent neuron damage which often leads to death of an individual. If a patient survives a traumatic brain injury, there is often permanent damage

inhibiting completion of daily tasks (Gatson et al. 2012). After a traumatic brain injury, nerve cells undergo oxidative stress and inflammation which may ultimately lead to apoptosis. Women are more likely to recover from a traumatic brain injury leading researchers to investigate hormones as an effective source of neuron protection (Gatson et al. 2012). Research investigating estrogen's effect on neuronal recovery after a traumatic brain injury was conducted by Gatson et al. (2012). Male rats were induced with a traumatic brain injury by a small incision in the skull. The rats were then given 0.5 mg/kg of estrone. Brain matter and lesions were then analyzed. The results of this study indicated a significant protective effect on the neurons in the parietal cortex and hippocampus in the groups that were given the estrone. Thus, this research suggests estrone may be useful in protecting neurons of individuals who experience traumatic brain injury

Johnsen and Murphy (2011) dissected male and female rat brains to collect cortical neurons. Cultured neurons were treated with 17β-estradiol and isoflurane prior to oxygen and glucose deprivation. Isoflurane is a halogenated ether used as an inhaled anesthesia. A gas exchange system was used to administer isoflurane to cells at 0% and 3% for one hour. Afterwards, cells were returned to normal conditions and deprived of oxygen and glucose for two hours the following day. Isoflurane protected male and female neurons from oxygen and glucose deprivation. Estradiol alone protected female neurons from oxygen and glucose deprivation. Seventeen β-estradiol was found to increase viability of cells. In addition, female neurons were found to be less affected by stressors compared to the male neurons.

There has been conflicting research on the effects of estrogen use. Marriott and Wenk (2004) suggested the timing of taking estrogens is essential to the protection of neurological diseases. For instance, taking estrogens before the onset of Alzheimer's or dementia may protect

neurons from deteriorating (Marriott and Wenk 2004). In addition to timing, correct dosage that mimics the natural cycle of fluctuating hormones is essential and may slow down the progression of Alzheimer's disease in postmenopausal women (Marriott and Wenk 2004). Research has found estradiol, specifically 17β-estradiol, provided significant protection to neurons after rats were induced with spinal cord injuries and global cerebral ischemia (Mosquera et al. 2014; Raval et al. 2009). Less is known about nonendogenous estrogens, and some research suggests the use of any estrogen imposes more risk on an individual. Brunner et al. (2010) treated women with CEEs in a one year trial. Although women who consumed CEEs showed a reduction in vasomotor symptoms and vaginal dryness, associated symptoms returned after stopping CEEs. The Women's Health Initiative study was a set of clinical trials and was an observational study that began in 1991 and lasted for 15 years with over 161,800 overall healthy postmenopausal participants. The study found participants taking estrogens with progestin had increased incidences of coronary heart disease, stroke, and pulmonary embolism compared to participants that did not take the HRT. This research was stopped early due to the increase incidence of invasive breast cancer among participants.

Hormone Replacement Therapy

Hormone replacement therapy (HRT) is a combination of estrogens and progestin that is used to relieve menopausal symptoms such as night sweats, hot flashes, bone loss, and mood swings by providing hormones which are no longer produced at the same level in the recipient's body. HRT first became available in 1940, and it is still an approved method used today by many women. Although some estrogen replacements can be purchased over-the-counter, HRT is often prescribed by a physician in the oral form. Common HRTs include Prempro (0.3 mg to 0.625 mg conjugated estrogens and 1.5 mg to 5 mg medroxyprogesterone from Wyeth

Pharmaceuticals), progestogen (ranges from 100 mg to 400 mg from in the pill form and 4% to 8% in gels from Elfin Pharmaceuticals), and Premarin (conjugated estrogens 0.3 mg to 0.625 mg administered orally for three weeks with one week off from Pfizer Pharmaceuticals). The latter includes ten estrogen compounds, some of which are outlined in the following section (Zhao and Brinton 2006). Connelly et al. (2000) measured the prevalence and duration of women taking HRT from 1990 to 1995. They found menopausal women ages 50 to 54 years were the greatest users of HRT (24%). Less than 5% of women 75 years and older used HRT. Many women discontinued HRT after a year.

Once HRT is taken, hormones enter the body and bloodstream where they then act on corresponding receptors. Health benefits such as prevention of osteoporosis and improved sleep leading to an overall higher quality of life are linked with estrogens present in HRT. Yet, it is common for vasomotor side effects associated with menopause and post-menopause to return after stopping the HRT as these hormones will decrease in the body once no longer consumed regularly (Brunner et al. 2010). Additionally, risks such as incidences of cancers increase as one takes HRT (Hendrix et al. 2006).

Conjugated Equine Estrogens

In addition to progestins, estradiols, and estrone, conjugated equine estrogens (CEE) are also commonly used in HRT (Zhao and Brinton 2006). CEEs include several estrogens that are not endogenous to humans. The composition of Premarin includes the following, listed in order of decreasing concentration: sodium estrone sulfate, sodium equilin sulfate, sodium 17α-dihydroequilin sulfate, sodium 17α-estradiol sulfate, sodium 8,9-dehydroestrone sulfate, sodium equilenin sulfate, sodium 17β-dihydroequilin sulfate, sodium 17α-dihroequilenin sulfate, sodium

17β-estradiol sulfate, sodium 17β-dihydroequilenin sulfate, and sodium 8, 9-dehydroestradiol sulfate (Figure 1a) (Zhoa and Brinton 2006). Equilenin, also known as 6,8-didehydroestrone and estra-1,3,5,6,8-pentaen-3-ol-17-one, has a molecular formula of $C_{18}H_{18}O_2$ whereas estradiol has six more hydrogens making its chemical composition $C_{18}H_{24}O_2$ (Figure 1). Estrone has a chemical composition of $C_{18}H_{22}O_2$ (Figure 1c). Although estrone, 17β-estradiol and equilenin are all present in Premarin, the relative amount of each differs. Estrone accounts for approximately 49% while equilenin accounts for 2.2%, and 17β-estradiol only accounts for 0.9% of the estrogen concentrations. Similar to Premarin, Prempro is composed of a combination of estrogens comprised of 20% conjugated estrogens and 80% medroxyprogesterone (pregn-4-ene-3, 20-dione, 17-acetyloxy-6-methyl-6α). Synthetic steroid hormones, such as progestins, are also commonly used. In addition to equilenin being a nonendogenous estrogen as opposed to estradiol, equilenin has a double bond present on its compound whereas estradiol does not. Equilenin can bind to estradiol receptors. However, the affinities differ. Enzymes to process CEEs are absent in the human body. Thus, CEEs stay longer in the body as opposed to human estrogens (Hendrix et al 2006). Estrone, estradiols, and progestins have been found to provide protection to neurons in individuals who have had a stroke (Hsieh et al. 2012). Yet, CEEs have been found to offer less protection, and studies have indicated CEEs may carry more risk compared to endogenous estrogens (Brunner et al. 2010; Grimes and Hughes 2015).

Chemical structures of estrogens may have a role in the protective effects they offer. Certain estrogens in Premarin provide a mechanism of protection for neurons when induced with stress. However, not all estrogens in Premarin provided protection (Zhao and Brinton 2006). This study used β-amyloid, a toxin associated with the brains of Alzheimer's patients. The sixth leading cause of death, Alzheimer's disease results in loss of neurons and degeneration of brain

matter. When β-amyloid misfolds, aggregates and toxic plaques form which are called β-amyloid plaques. These plaques can lead to neuronal cell death (Pujol-Pina et al. 2015). Recent studies have found a positive relation between oxidative stress and β-amyloid protein (Porcellotti et al. 2015). This is an important implication suggesting further research in protecting the neurons from oxidative stress thereby preventing β-amyloid plaques and ultimately inhibiting Alzheimer's disease is needed. Zhao and Brinton (2006) tested different concentrations of estrogens as possible neuron protectors. Basal forebrain neurons were collected at embryotic day 18. Neurons were treated with CEEs. While the combination CEE was found to protect the neurons, the researchers wanted to determine which of the hormones in the CEE were most effective at providing neuronal protection toward the neurons. They repeated their study with known quantities of the specific estrogens. The estrogens used included those that are produced naturally in the ovaries of humans and nonendogenous estrogens which were the following: estrone, 17α-estradiol, 17β-estradiol, equilin, 17α-dihydroequiline, 17β-dihydroequiline, equilenin, 17α-dihydroequilenin, 17β-dihydroequilenin, and dehydroesterone. The neurons were first treated with the estrogens and then exposed to the β-amyloid protein. Although all the hormones provided some level of protection to the neurons, 17β-estradiol, estrone, and dehydroestrone provided the most neuronal protection. Thus, two endogenous estrogens (17β-estradiol and estrone) and one nonendogenous (dehydroestrone) which is found in horses like equilenin, provided a significant protection to neurons.

Risks Associated with Estrogens

The benefits and risks of hormones to the body are essential to understand when one is choosing a hormone replacement therapy. Brunner et al. (2010) analyzed over 10,000 postmenopausal women prior to and following hormone replacement therapy. A decrease in

postmenopausal symptoms, such as vasomotor symptoms, were reported by women taking HRT. However, women taking HRT also reported breast tenderness (Brunner et al. 2010). Additionally, side effects were likely to significantly increase once the women discontinued the use of CEE (Brunner et al. 2010). This is an important aspect to consider when choosing a hormone replacement therapy since HRT should not be used long-term due to the increased risk of breast, endometrial, ovarian, and uterine cancers (Tozzi et al. 2015). The Women's Health Initiative study concluded postmenopausal women who had long-term exposure to hormone replacement therapy which included estrogens and progestin had a 26% higher incidence of breast cancer as well as an increased risk of heart attacks and associated cardiovascular health problems. This study was stopped early due to the incidence of increased breast cancer in women. Participants taking HRT also had an increased with of cardiovascular disease. However, this study indicated the combination of equine estrogens with progesterone lowered the risk of colorectal cancer. Additionally, younger women were less likely to have adverse side effects in this study. Harlan et al. 1993 used 689 women of different races in their study and found white women were more likely to have estrogen receptor-positive tumors as opposed to African American women. Thus, demographics is an important aspect to consider which plays a significant role as well as the health and age of the individual taking estrogens. Although there is conflicting research about the risks associated with estrogen's effect, many research still suggests estrogen use may be beneficial when taken at the proper time and proper dosage.

The role of various stressors used to evaluate the effect of estrogens has not been widely studied and the few studies that have been done show conflicting results. For instance, Grimes and Hughes (2015) found cellular protection when rat astrocytes were stressed with hydrogen peroxide whereas Zhao and Brinton (2006) found selective estrogens in Premarin provide

protection when rat neurons were stressed with β-amyloid protein. The actual mechanism of estrogen that takes place to provide protection is unknown. However, receptor blocking research may offer insight. Additionally, different stressors such as hydrogen peroxide, cortisol, epinephrine, and low oxygen concentration may elicit different effects in cells. Less research is available pertaining to astrocytes stressed with epinephrine and cortisol. However, previous research has found some stressors elicit other stressors. Chen at al. (2014) found hypoxic environments to increase the release of cortisol resulting in damage to astrocytes and cerebral edema. Below is a review of three main stressors that are typically used in testing the efficacy of estrogen on cell growth and proliferation.

Stressors

Epinephrine

Epinephrine and norepinephrine are produced in many organisms and categorized as both neurotransmitters and hormones. They act as neurotransmitters when released in the brain and act on receptors in the brain. When referred to as hormones, they are released from a neuron and travel in the bloodstream where they bind to receptors on other cells. Epinephrine primarily comes from the adrenal medulla whereas norepinephrine primarily comes from the brain. Dihydroxyphenylalanine (DOPA) is synthesized from tyrosine by DOPA decarboxylase. Dopamine-β hydroxylase synthesize dopamine resulting in norepinephrine. Phenylethanol amine N-methyl-transferase is the enzyme that synthesize epinephrine from norepinephrine (Purves et al. 2012). Structurally, epinephrine and norepinephrine are similar except epinephrine has an additional methyl group (Figure 2). When epinephrine is released, it binds to a class of G protein-coupled receptors known as adrenergic receptors causing various response in other cells

(Strosberg 1993). Adrenergic receptors are divided into two main groups, α and β where they can then be further divided by subtypes (α_1, α_2, β_1, β_2, β_3). Adrenergic receptors often act in the brain, but they are also located throughout the body from the muscles in the eye to the urinary sphincters (Qin et al. 2015). Alpha receptors are located on the arteries, live cells, platelets, and blood vessels of smooth muscles. When epinephrine or norepinephrine is released, they bind to the alpha receptors in the arteries resulting in increased blood pressure due to a constriction in the arteries. Beta receptors are located on the heart and bronchioles of the lungs as well as arteries of skeleton muscles (Strosberg 1993). The autonomic nervous system (ANS), which consists of the sympathetic and parasympathetic nervous systems, is responsible for controlling the visceral motor response. The ANS regulates organs and bodily functions and the response is dependent upon which nervous system, sympathetic or parasympathetic, is activated. When the sympathetic nervous system is activated under conditions of stress, higher concentrations of epinephrine and norepinephrine are released (Liao et al. 2015). This state is commonly referred to as 'fight-or-flight' which is characterized by increased in blood pressure, heart rate, sticky/thick saliva, release of glucose, increased blood flow to skeletal muscles, and dilated pupils (Vasunilashorn and Cohen 2014). Although both epinephrine and norepinephrine are needed in the body, overproduction of these catecholamines may lead to cell death due to extended oxidative stress encountered by the cells (Liao et al. 2015). Previous research has indicated over production of epinephrine and norepinephrine have a positive correlation with increasing tumors, spread and growth of cancers, damaging neurons resulting in neuronal diseases, and inducing neuronal cell death (Qin et al. 2015).

It is expected there would be a higher production of epinephrine and norepinephrine when an individual is in a stressful situation since stress is positively correlated to these

catecholamines. One study showed the significant health factors an individual may suffer when activation of the sympathetic nervous system is extended (Teleger et al. 2015). This study consisted of 131 individuals (82 women and 49 men) with a variety of health conditions. The effects of induced sympathetic nervous system versus parasympathetic nervous system on the cardiovascular function were investigated. The researchers found a significant decrease in cardiovascular health in individuals by calculating heart rate variability and baroreflex sensitivity when the sympathetic nervous system was induced over an extended time (Teleger et al. 2015).

Weiming et al. 1998 analyzed calcium influx, cytoplasmic and mitochondrial reactive oxygen species and found cultured hippocampal neurons had increased vulnerability when cells were exposed to catecholamines at concentrations of 10-200 μM. When catecholamines were combined with amyloid-β-peptide, its detrimental effects to the cells increased. Neurons that received higher concentration (100-200μM) resulted in death. This study also found certain antioxidants counteracted the effects of catecholamines and amyloid-β-peptide such as vitamin E, glutathione, and propyl gallate. Thus, vitamin E, glutathione, and propyl gallate could potentially be used as protectors against the catecholamines as stressors.

Liao et al. (2015) further demonstrated the risks associated with epinephrine and norepinephrine by testing catecholamine effects on the virus Enterovirus 71. The objective in the study was to assess the percent of cells infected with the virus before and after the cells were exposed to the catecholamines. A Flow Cytometry Assay using rabbit anti-α1A ADR or β2-ADR and goat anti-rabbit antibodies was used to measure expression of adrenergic receptors in infected cells. The researchers found an increase percentage of viral-infected cells when the cells were treated with epinephrine and norepinephrine, indicating these catecholamines induced a negative stress on the cells where the healthy cells lost the ability to fight off the virus.

The impact of epinephrine and norepinephrine on stress is not confined to neuronal cells. Breast cancerous cells are also affected by levels of epinephrine and norepinephrine. One study tested the effects of these catecholamines on tumor progression and macrophages (Qin et al. 2015). They found a significant increase in growth of cancerous tumors when induced with epinephrine and norepinephrine. This study also found increased levels of epinephrine and norepinephrine has a specific negative effect on the immune system. As these catecholamine concentrations increased, macrophages decreased significantly leaving the immune system less effective at fighting infections, pathogens, and diseases such as cancerous tumors. This research of catecholamines further explains why a progressive growth in the cancerous tumor was observed once epinephrine and norepinephrine were induced (Qin et al. 2015).

The current study found it appropriate to use epinephrine as a stressor to astrocytes protected with and without estrogens to test the protected effects of different estrogens since much research correlates cellular stress/ death with high concentrations of epinephrine (Weiming et al. 1998; Qin et al. 2015).

Cortisol

A second stressor that is typically used in estrogen research is cortisol, a key regulator of stress response. Like epinephrine, higher concentrations are associated when individuals are under stressful conditions or in a stressful environment. Epinephrine and norepinephrine are first released followed by cortisol which effect last longer (Dindia et al. 2013). Cortisol consists of nine carbons, thirteen hydrogens, one nitrogen, and three oxygen atoms, and it is made when the hypothalamus senses a stressor (Figure 3). As a result, corticotropin releasing hormone stimulates the anterior pituitary gland to release ACTH molecules in the brain which travels to

the bloodstream. Cortisol production begins when ACTH reaches the adrenal cortex and stimulates cortisol (Mons and Beracochea 2016). The zona fasciculata of the adrenal cortex within the adrenal gland is where cortisol is formulated. Cortisol concentrations are typically highest early in the mornings and slowly decline throughout the day where concentrations are lowest in the evening and when one is sleeping. This pattern may be reversed for individuals who sleep during the day and are awake at late hours of the night. Cortisol levels typically range from 3 ud/dl to 20 ud/dl in a twenty-four hour timeframe (Dindia et al. 2013).

Although these concentrations of cortisol release are in the normal range and thought to be harmless, high concentrations and prolonged release has been related to detrimental effects in astrocytes including an increase in oxidative stress and a decrease in cellular viability (Chen et al. 2014). Parkinson's disease is characterized by chronic inflammation and an increase release of cortisol. This increase in cortisol release is thought to be related to dopamine neuron generation (Herrerero et al. 2015).

On a larger scale, high concentrations of cortisol are known to interfere with cognitive function, increase one's weight and blood pressure as well as increase the risk of certain diseases (Jackson et al. 2017; Cozma et al. 2017). One study tested the cortisol concentrations of prepubescent girls and included participants who were normal weight and obese. Scalp hair was chosen to analyze for cortisol levels rather than blood samples because hair provides an index of long-term circulating cortisol. Salivary samples were also collected from participants. The study concluded there was a positive correlation with high concentrations of cortisol in scalp hair samples and salivary samples of obese participants compared to normal weight participants (Papafotiou et al. 2017).

Additionally, Jackson et al. (2017) used human hairs to determine the amount of cortisol present and found a positive correlation between obesity and high concentrations of cortisol. Dindia et al. (2012) exposed rainbow trout cells to cortisol *in vitro* and found cortisol exposure impacted elasticity of hepatic plasma membranes, fluidity, and surface topography. Other research found hydrocortisone inhibited proliferation of human osteoblast cells (Tsunashima et al. 2011).

Anoxia

Low oxygen concentration, anoxia, is another source of stress cells in the brain can encounter; if this stress is extended, neuronal death will occur (Xie et al. 2014). Stroke victims suffer from low oxygen concentrations which can damage many organs and be detrimental if neurons are not able to get the oxygen needed to properly function. Strokes result from a clot, blockage, or rupture preventing the regular flow of oxygen in the body to neurons. As a result, neurons located in affected areas of the brain undergo anoxic stress and this affects surrounding tissues (Pabon et al. 2014). The amount of time the neurons are deprived of oxygen and an individual's health are two factors that determine the level of neuronal damage and oxidative stress neurons encounter (Xie et al. 2014). The motor cortex controls the preparation and execution of motor control. Prior research illustrated progesterone and estrogens provided a protective mechanism for neurons when model strokes were induced (Yousuf et al. 2014).

Strokes contribute to a number of deaths every year. If a stroke affects the motor cortex, neurons in this region of the brain would be damaged; thus, motor control would significantly decline (Bajaj et al. 2015). Individuals who survive a stroke often suffer permanent damage from free radicals due to anoxic stress such as inability to move limbs on one side of the body (Pabon

et al. 2014). The protective effects of ischemic post conditioning are essential for individuals who have suffered from a stroke to prevent damage to neurons and other vital cells as well as organs (Xie et al. 2014). Certain hormones such as 17β-estradiol and progesterone provided neuronal protection after an individual suffered from a stroke. In a study using middle-aged rats who suffered post-stroke brain infarction and functional deficits, different levels of progesterone (8 mg/kg, 16 mg/kg, and 32 mg/kg) were injected through the peritoneum after cerebral ischemia was induced at the right middle cerebral artery occlusion. Motor, sensory, and cognitive tests were used to determine recovery post-stroke during intervals up to 22 days. Their results indicated that all doses were effective at reducing the effects of ischemic infarct injury, but the rats who received 8 mg/kg of progesterone suffered from the least side effects (Yousuf et al. 2014).

Estrogens are known to have a positive role in the health of the cardiovascular system, but less is known about their effects and relation to ischemic strokes. A study was conducted that used 305 patients who were younger than 50 years old and suffered from an ischemic stroke. Blood tests were collected from patients and analyzed for hormone levels. The results indicated that lower levels of estradiols caused a significant increase in an individual's risk of experiencing an ischemic stroke (Hsieh et al. 2012). In contradiction, the Women's Health Initiative study found women taking HRT had higher incidences of cardiovascular diseases including strokes. Previous research has indicated neuronal protection after an acute stroke in both male and female rats when treated with estrogens during the therapeutic window period which is often three to six hours. However, this period may be extended for humans (Simpkins et al. 2004). Estrogen analogues were used to treat both male and female rats following induced ischemic stroke. The analogues reduced feminization but were still potent in neuron protection (Simpkins et al. 2004).

Since this research utilized both males and females, HRT may be beneficial to both men and women. Since previous research found estrogens to protect neurons and cortisol is known to affect neurons and astrocytes negatively, astrocytes were stressed with cortisol after cells were pretreated with estrogens to test the protective effects of different estrogens.

The current research examined the effects on astrocytes when exposed to various stressors after being treated with a single estrogen or a combination of estrogens. The hormones used in this research included equilenin, 17β-estradiol, and a combination of the two. Analyses were conducted to determine if a certain estrogen treatment offered more protection to the human astrocytes compared to other treatments.

Hypotheses

1.) The astrocytes treated with 17β-estradiol will have the most significant astrocyte protection for the stressors epinephrine or cortisol. Increased viability of astrocytes will be seen as concentrations of estradiol increases.

2.) The estrogen combination 17β-estradiol with equilenin will have greater cellular protection compared to cells treated with the single equilenin alone, but less cellular protection will be seen in the hormone combination compared to cells treated with 17β-estradiol when the stressor is epinephrine or cortisol.

This hypothesis therefore indicates the astrocytes treated with 17β-estradiol will have greater cell viability compared to cells, and cells treated with equilenin alone were expected to have lower cell viability

3.) The estrogen treatment, 17β-estradiol, will provide more protection to astrocytes when the stressor is low oxygen concentration compared to equilenin treatments. Higher viability of astrocytes will be seen as concentrations of estradiol increases.

4.) The hormone combination, 17β-estradiol with equilenin will provide the cell with greater neuronal protection as opposed to the single equilenin treatment, but less cellular protection will be seen compared to the single 17β-estradiol which will have the highest cell viability when the stressor is low oxygen concentration.

5.) The cells treated with estrogens will have higher cell viability than the controls.

METHODS

Cell Culture

Human astrocytes (cell line 1321N1) were purchased from Sigma-Aldrich. These cells originated from a brain astrocytoma and have glial cell morphology. The cells were thawed and grown in a T-75 mL flask with Dulbecco's Modified Eagle's Medium (DMEM purchased from Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 5% antibiotic/antimycotic (Sigma-Aldrich), and 2 mM L-glutamine. The cells were grown in an incubator at 37°C and 10% carbon dioxide (Grimes and Hughes 2015). These conditions were used in all treatments unless otherwise noted. Cell growth was monitored under an inverted light microscope. Once approximately 80% confluency was reached, cells were subcultured using 0.25% trypsin-EDTA and plated in 96-well plates at a concentration of $1x10^6$ cells/mL. A hemocytometer was used to assess viability using the Trypan Blue dye eye vision method. A 1:1 of cell suspension and 4% trypan blue was used to do this. Samples that contained more dyed cells indicated a higher concentration of cells, whereas clear samples indicated a low concentration of cells using the

hemocytometer. Each well in the 96-well plate contained 200 μL of media. Once plated in the wells, treatments began the next day.

Estrogen Treatment

Both stock estrogens (17β-estradiol and equilenin, Sigma-Aldrich) were stored at 4°C and prepared the same day of treatment. Serial dilutions in PBS were performed to dilute the stock estradiol and equilenin. Cells were pretreated with estrogens for one hour before stressor exposure. Estrogens used in this study included 17β-estradiol, equilenin, and a combination of the two. Cells were treated with estrogens varying in concentration from 10 nM to 10 μM (10 nM, 100 nM, 1 μM, and 10 μM) (Grimes and Hughes 2015). Controls received the same volume of vehicle (phosphate buffer saline) as estrogen treatments.

Preliminary research took place using a Muse Cell Analyzer to perform a flow cytometry assay to analyze cells. This machine uses a laser-based fluorescence detection which evaluated cellular parameters in order to deliver quantitative data on cells. After cells were cultured using both positive and negative controls, 1X Assay Buffer was added to cells at $1x10^6$ cells/mL for incubation with Muse Oxidative Stress working solution. After cells were treated with the reagent, the cells were incubated for thirty minutes at 37°C. Cells were analyzed with an adjusted gateway (this was used as a baseline) to determine oxidative stress by measuring total cell count and percentage of reactive oxidative species. Results that were above this adjusted gateway were considered to be reactive oxidative species. Thus, these cells were considered to be under oxidative stress. The cell analyzer measured ROS positive species in percentages. The higher percentage of ROS positive cells, the more cells undergoing oxidative stress.

Epinephrine as a Stressor

The first objective of this research was to determine if a single or combination of estrogens at various concentrations applied prior to 100 μM epinephrine exposure affects astrocyte viability. Human astrocytes were cultured and plated in an incubator for twenty-four hours. They were then treated with 17β-estradiol or equilenin or a combination of the two at concentrations of 0nM (controls), 10 nM, 100 nM, 1 μM, and 10 μM. After one hour estrogen exposure, the astrocytes were exposed to 100μM epinephrine for one hour. Astrocytes received post-stressor treatment after epinephrine was removed and allowed to sit in estrogens until they were analyzed using a Muse Cell Analyzer for flow cytometry and an MTT assay the following day to detect cell viability, proliferation, and oxidative stress.

Preliminary research was done to determine the optimal concentration of epinephrine to induce measurably oxidative stress in the cells. The trials included 1 μM, 10 μM, 100 μM and 1 mM, and exposure time varied from one hour to two hours (Grimes and Hughes 2015). Following results of the initial study, 100 μM with an exposure time of one hour was determined to be the appropriate treatment used to induce oxidative stress analyzed by using the Muse Cell analyzer. The Muse Analyzer measured reactive oxidative species to determine cells under stress (Mao et al. 2004). This concentration of epinephrine, 100 μM, was also used for the MTT Assay.

Following one hour pretreatment of estrogens, cells were exposed to 100 μM epinephrine for one hour. Media was then replaced to remove the stressor. Treated cells were retreated with estrogens as indicated above. Thus, treated cells (excluding controls) received estrogen pre- and post- stressor exposure. All cells were analyzed using an MTT Assay to measure cell viability consisting of seven trials at an absorbance of 570 nm to detect cell viability.

Cortisol as a Stressor

Preliminary research was done to determine the optimal cortisol concentration to stress cells as indicated by decrease in viability. Cells exposed to stressor were compared to controls that did not receive the stressor. Concentrations included 10 nM, 100 nM, and 1 μM with exposure times of one hour, for hours, and twenty-four hours (Anacker et al. 2013; Tsunashima et al. 2011). One micro molar concentration of cortisol with an exposure time of one hour was determined to be the most consistent in achieving the best acute stress resulting in a decrease in cell viability. Cells were plated in 96-well plates and returned to the incubator at 37°C and 10% CO_2.

The following day cells were pretreated with the estrogens and concentrations listed above for one hour. Afterwards, cells were exposed to 1 μM cortisol for one hour and then immediately analyzed for cell viability using an MTT assay consisting of seven trials at an absorbance of 570 nm to detect cell viability.

To determine if a single or combination of estrogens at various concentrations applied prior to cortisol exposure affects astrocyte viability, human astrocytes were cultured and plated in an incubator for 24 hours. They were then treated with a single estrogen treatment of 17β-estradiol or equilenin at concentrations of 0 nM (controls), 10 nM, 100 nM, 1 μM, and 10 μM. After one hour estrogen exposure, the astrocytes were exposed to 1 μM cortisol for one hour. Following stressor exposure, cells were analyzed using an MTT Assay to detect cell viability.

Anoxia as a Stressor

To determine if a single or combination of estrogens at various concentrations applied prior to anoxia exposure affects astrocyte viability, human astrocytes were cultured and placed in

the incubator for 24 hours at a standard air flow. Cells were treated with a single estrogen (17β-estradiol or equilenin) or a combination of the two for one hour. Following estrogen pretreatment, cells were exposed to a hypoxic environment for one hour at 37°C. An MTT Assay was conducted immediately after one hour hypoxic exposure consisting of six trials at an absorbance of 570 nm to detect cell viability.

Cells were plated in 96-well plates and returned to the incubator at 37°C, 10% CO_2, and standard air flow. Astrocytes were pretreated with the estrogens at varying concentrations listed above for one hour. The cells were then placed in an incubator at 37°C without air flow or CO_2 for one hour. The hypoxic condition of the incubator was achieved by lighting multiple matches until a flame was no longer produced which was an indication oxygen levels were lowered in the incubator (Johnsen and Murphy 2011). Immediately following one hour exposure to the hypoxic incubator, cells were analyzed using an MTT assay consisting of six trials at an absorbance of 570 nm to detect cell viability.

MTT Assay

The MTT assay measures cell viability by assessing the rate of cellular metabolism using a color test. The MTT assay kit was purchased from Sigma-Aldrich (Stock No. TOX-1 and catalog No. M-5655 for kit components). The media in all wells was replaced with 100 μL media. Blanks served as controls and received the same volume of reagents and solutions as treatments. Ten microliters of an MTT reagent (3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide), was added to each well to produce a final concentration of 10%. The MTT reagent is reduced by cellular oxidoreductase present in living cells and forms an insoluble formazan indicated by a purple color. The 96-well plate was then incubated for two hours at

37°C and 10% CO_2. Following incubation, 100 µL of MTT Solubilization Solution was added to each well to dissolve the crystal in formazan product. A microplate spectrophotometer was used to measure absorbance at a wavelength of 570 nm. Higher absorbance values indicated higher cell viability when comparing treatments to controls.

Statistical Analysis

Statistical software including JMP (from SAS) and 17.0 SPSS were used to analyze data collected from the seven trials using epinephrine and cortisol as a stressor and the six trials using anoxia as a stressor. A two-way ANOVA was performed on the epinephrine and cortisol trials, which analyzed the effect of stressor, estrogen concentration, and estrogen concentration by stressor. A test for normal distribution was performed on data. If a significance was reported, data was log transformed and log transformed data was analyzed using a two-way ANOVA. If a significance was found after conducting a two-way ANOVA test, a Tukey's Post-hoc test was performed to determine further information about the differences among the means across estrogen concentrations and presence/absence of stressor. A 95% confidence interval was used in this research. Thus, results that yielded p-values below 0.05 were considered significant. A one-way ANOVA was performed on data collected for the anoxia trials. In the epinephrine and cortisol trials, absorbance values obtained from the MTT assay were used as the dependent variable while estrogen concentrations and stressors were used as the independent variable. In the low oxygen trial, the dependent variable was absorbance values while the independent variable was estrogen concentrations.

RESULTS

Data are expressed in Tables 1 through 3 from the two-way ANOVAs and one-way ANOVA. Data are reported as absorbance value averages +/- standard error. The parameters used for the two-way ANOVA are listed in Tables 1 and 2 with the results. The parameters used for the one-way ANOVA are listed in Table 3 with the results.

Epinephrine

Astrocytes were pretreated with estradiol, equilenin, or a combination of the two for one hour following exposure to 100 μM epinephrine for one hour. Afterwards, astrocytes received estrogens again and were analyzed for viability using an MTT assay the next day. A two-way ANOVA was used to analyze astrocyte viability. All data in data tables are expressed as average optical density values at 570 nm +/- S. E. There was no significant difference between stressed and unstressed cells in terms of absorbance recordings across all estradiol treatments (Table 1, $p>0.05$). However, there was an estrogen treatment effect found (Table 1, $F=3.02$, $p=0.02$). The Tukey post-hoc test indicated 1 μM estradiol treatment yielded a significantly lower absorbance compared to the control treatments (1.13 +/- 0.21 and 0.20 +/- 0.19, respectively; Figure 4a). There was no significant difference between stressed and unstressed cells in terms of absorbance readings across all equilenin treatments and the controls ($p>0.005$). However, there was an estrogen concentration effect found (Table 1, $F=13.89$, $p<0.0001$). The Tukey post-hoc test indicated 10μM equilenin treatment yielded a significantly lower absorbance from all other treatments (control, 10nM, 100nM, and 1μM; 0.164+/-0.07, 0.92+/-0.07, 0.68+/-0.08, 0.66+/-0.08, and 0.66+/-0.08, respectively; Figure. 4b).

There was no significant difference between stressed and unstressed in terms of viability measurements across all estradiol with equilenin treatments and the controls ($p>0.005$). However, there was an estrogen treatment effect (Table 1, $F=7.66$, $p<0.0001$). The control treatment had a significantly high absorbance compared to the 10nM combination estrogen treatments 0.92+/- 0.08 and 0.57+/- 0.09, respectively. The combination of estrogen treatments at 1μM with and without stresses had a significantly lower absorbance compared to the control 0.52+/-0.09. The control treatment yielded a significantly higher absorbance compared to the 10μM combination estrogen treatment 0.92+/-0.08 and 0.26+/-0.09, respectively. The 10μM combination estrogen treatment was also significantly lower in absorbance from the 100nM combination estrogen treatment 0.26+/-0.09 and 0.63+/-0.09, respectively; Figure 4c).

Cortisol

Preliminary research took place using 10 nM, 100 nM, and 1 μM cortisol concentrations with exposure time of one hour, four hours, and twenty-four hours. Mean analysis of preliminary data helped draw the conclusion that 1 μM (average= 0.60) with an exposure time of 1 hour to astrocytes was the best treatment to use to lower cell viability (Figure 5).

A two-way ANOVA was used to analyze astrocyte viability after cells were stressed with 1 μM cortisol for one hour. All data are expressed as optical density values at 570 nm +/- S. E. Average absorbance values ranged from 0.82 to 0.53. There was no significant difference between stressed and unstressed estradiol treatments including controls. There was also no significant difference found across all estradiol treatments including controls. Thus, there was no estrogen effect ($p>0.05$; Figure 5a, Table 2, $F=1.29$, $p=0.28$).

There was no significance between stressed and unstressed equilenin treatments including controls. However, there was an estrogen concentration effect (table 2, $F=4.01$, $p<0.0001$). The equilenin 10μM treatment, regardless of stressor, yielded a significantly lower absorbance value compared to the control, 10 nM, and 100 nM (0.448 +/-0.10, 0.712 +/-0.09, 0.684 +/- 0.120, 0.77 +/-0.101 respectively; Figure 5b). The 1 μM and 10 μM equilenin treatments did not significantly differ ($p>0.05$).

There was no significant difference between stressed and unstressed estradiol with equilenin treatments including controls ($p>0.05$). However, there was an estrogen concentration effect (Table 2, $F=9.89$, $p<0.0001$). The control, 10 nM, 100 nM and 1 μM combination estrogen treatments did not significantly differ from one another ($p>0.05$; Figure 5c). However, the 10 μM combination estrogen treatment had a significantly lower absorbance from the control, 10 nM, 100 nM and 1 μM treatments (0.227+/-0.092, 0.712 +/- 0.078, 0.721 +/- 0.098, 0.513 +/- 0.099, 0.655 +/-0.092, respectively; Figure 5c).

Oxygen

A one-way ANOVA was used to analyze viability after the astrocytes were stressed in a hypoxic environment. All data are expressed as optical density values at 570 nm +/- S. E. No significant difference was found across all three estrogen treatments ($p>0.05$; Figure 6). The control stressed yielded an average absorbance of 0.53. Astrocytes treated with estradiol yielded average absorbance values ranging from 0.58 to 0.53, and there was no significant difference in viability of astrocytes across estradiol concentrations ($F=1.61$, $p=0.178$; Table 3, Figure 6a). Equilenin treatments yielded average absorbance values ranging from 0.48 to 0.63, and there was no significant difference in viability of astrocytes across equilenin concentrations ($F=1.83$,

p=0.127; Table 3, Figure 6b). Estradiol with equilenin treatments yielded average absorbance values ranging from 0.43 to 0.63, and there was no significant different across concentrations (F=1.9, p=0.112; Table 3, Figure 6c).

DISCUSSION

Although there was not a significant difference between the stressed and unstressed treatments, estradiol did not decrease cell viability (with the exception of 1 μM estradiol treatment in the epinephrine trials). Higher viability of astrocytes was not seen as concentrations of estradiol increased when cells were stressed with cortisol and epinephrine. Therefore, this hypothesis was rejected. No protection was indicated by data; therefore, hypotheses were rejected. If data had indicated certain estrogen treatments increased cell viability, the protection would have been secondary as glial cells are responsible for maintaining the blood brain barrier among other functions. The hypothesis that the estrogen combination 17β-estradiol with equilenin will have greater cellular protection compared to cells treated with the single equilenin alone when the stressor is epinephrine or cortisol was rejected. The hypothesis that 17β-estradiol alone will provide a better protective mechanism when the stressor is low oxygen concentration compared to equilenin alone and estradiol with equilenin was rejected. Additionally, the hypothesis that higher viability of astrocytes will be seen as concentrations of estradiol increased was rejected. The hypothesis that the hormone combination, 17β-estradiol with equilenin, will provide the cells with greater neuronal protection as opposed to the single equilenin treatment when the stressor was low oxygen concentration was also rejected. No estrogen treatment demonstrated protective effects. All estrogen treatments yielded lower cell viability as opposed to the controls. Additionally, many treatments yielded significantly lower cell viability compared to the controls, indicating the estrogens had deleterious effects. These detrimental effects were

evident in higher concentrations, regardless of the presence of stressors. Previous research found estrogen to be detrimental as did the current study. For instance, the Women's Health Initiative study found estrogens to be dangerous in increasing the incidence of cancers, especially in older women. Each experiment set is discussed below.

Epinephrine

Overall, estradiol pretreatments did not protect the human astrocytoma cell line. Interestingly, exposure to 1 μM estradiol resulted in reduced cell viability, regardless of epinephrine presence. This differs from Grimes and Hughes (2015) findings that found higher concentrations of estradiol provided astrocytes with more protection compared to lower concentrations of estradiol and controls. This also differs from Zhao and Brinton (2006) who found higher concentrations of endogenous estrogens (estradiol and estrone) provided significant neuronal protection. With the exception of 1 μM estradiol treatment in the current research, the astrocytes treated with estradiol did not indicate a lower absorbance in the MTT assay whereas higher concentrations of equilenin alone and the combination of estradiol with equilenin had lower absorbance indicating lower cell viability. The results did not support previous research that indicated 17β-estradiol provided astrocyte protection compared to cells not treated with 17β-estradiol. Grimes and Hughes (2015) found lower concentrations of estradiol had lower cell viability compared to higher concentration of estradiol (10 μM) significantly increased cell viability for both stressed and unstressed astrocytes. However, Grime and Hughes (2015) used mouse astrocytes in their research whereas the current research used human astrocytes. Thus, differences in phylogenetic source of the cell lines may play a role in variation of results. The current research used a human cell line, which is beneficial in providing information of the effects estrogens have on cellular damage to women consuming estrogens. Other research

outside the brain have found estradiol to provide protection. For instance, May et al. (2006) tested the protective effects of estradiol in rat pancreatic β-cells after recognizing the low prevalence of diabetes in females which suggest a sex hormones may have a role in protection of β-cells. This study found estradiol offered significant protection to β-cells preventing oxidative injury and diabetes mellitus in both male and female rats. Thus, estradiol's protective effects may be offered throughout the body and not just confined in the brain as neuroprotection. Additionally, May et al (2006) measured oxidative stress whereas the current research measured cell viability. Since 1 μM estradiol significantly decreased cell viability, but the highest concentration of estradiol (10 μM) did not raises further questions. Such questions include if these results are specific to the cell line that was used in the current research or if an unnoticed error occurred in the research that yielded these results. Assuming the data are correct, it can be hypothesized that 1 μM is detrimental to the cell line used whereas 10 μM is less detrimental when cells are treated with estradiol.

The cells pretreated with equilenin, a conjugated equine estrogen, showed no protective mechanism compared to controls. The highest equilenin concentration, 10μM, showed a significant decrease in cell viability compared to controls, indicating a detrimental effect on the cells regardless of epinephrine treatment (Figure 4b). Although other studies found equilenin to provide cellular protection when cells were exposed to stressors for a short time, the current research did not (Grimes and Hughes 2015). Similar to this research, other studies have found CEEs offer less protection to cells compared to 17β-estradiol and may impose dangers (Brunner et al. 2010). The results of the current research suggest high concentrations of CEEs may not only provide no protection to astrocytes, but may also be detrimental to cells. Thus, taking CEEs may be detrimental to cells in the brain.

Comparable to the results found when cells were treated with the high concentration of equilenin alone, the combination of the estrogens showed no protective mechanism against the epinephrine stressor. In fact, the 10 nM, 1 μM and 10 μM combination estrogen treatments had significantly lower cell viability regardless of epinephrine exposure compared to both controls (Figure 2c). The combination of estrogens used was chosen to illustrate the combination of estrogens that is consumed when taking HRTs that have CEEs. However, Premarin has more estrogens than the combination used in the current study. Zhoa and Brinton (2006) found combined estrogen treatments to provided significant protection compared to single estrogen treatment when rat astrocytes were stressed with glutamate. In contrast, The Women's Health Initiative study found similar results to the current research indicating CEEs may be harmful and combining estrogens may be detrimental to cells as well.

Treatment of epinephrine did not affect cell viability as measured by the MTT assay. A higher concentration of epinephrine is released during activation of the sympathetic nervous system or often referred to as 'fight or flight.' Individuals who are stressed have higher levels of epinephrine in their bodies which can increase damage to astrocytes while taking CEEs (Liao et al. 2015). Initial research using flow cytometry with epinephrine concentrations ranging from 1 μM to 1 mM found 100 μM to invoke the most oxidative stress. This concentration exhibited a higher percentage of cells undergoing oxidative stress while cell viability was consistent. The MTT assay used to analyze cells only indicated relative cell viability and proliferation, not oxidative stress as measured by flow cytometry assays.

The concentration of estrogens chosen in this research illustrated the actual concentration that would be present at an *in vivo* level. If an individual is suddenly affected by stress, the sympathetic system activates, and a higher concentration of epinephrine is released. Normal

range of epinephrine is 10 pg/mL to 30 pg/mL, but this number increases when an individual is stressed (Purves et al. 2012). Thus, if a woman is taking HRT with CEEs present and is undergoing such stress, damage may occur to astrocytes. Since astrocytes play a significant role in the brain such as maintaining ion concentration and synaptic support, neurological functions may decrease (Purves et al. 2012). A significant decrease in viability of cells treated with epinephrine was not found. Consequently, in the future, a higher concentration of epinephrine or a different exposure time may need to be examined.

Cortisol

Since higher levels of cortisol are related to the body's long-term stress exposure affecting overall health as well as cognitive function, 1 μM of cortisol concentration was chosen to mimic cortisol's effect *in vitro* (Jackson et al. 2017; Cozma et al. 2017). When conducting the preliminary trials to determine the adequate concentration of cortisol as well as best exposure time that would induce stress and decrease cell viability, it was noted that cells treated with cortisol for four hours and twenty-four hours may have overcome stressor and thus proliferation continued during extended stressor exposure time (Figure 5). Thus, 1 μM of cortisol for one hour was chosen as the best stressor.

Pretreatment with 17β-estradiol offered no significant cellular protection to the cortisol stress, regardless of concentrations. However, it was not detrimental either. Pretreatment with equilenin offered no significant cellular protection to the cortisol stress. The highest concentration, 10 μM, of equilenin alone and equilenin with estradiol had a significant lower cell viability regardless of cortisol exposure. These findings are similar to the epinephrine stressor. The results gathered from this research are important to consider for women who are considering

taking HRT that have non-endogenous estrogens in it. Since estradiol is naturally produced in the body and conjugated equine estrogens include endogenous estrogens in them as well, this combination treatment reflects the combination of estrogen that would be present in the body if an individual is taking such hormone therapy (Zhao and Brinton 2006). Additionally, the impact of stress may increase the cellular detrimental effects when CEEs are administered at higher concentrations.

Grimes and Hughes (2015) used mouse astrocytes and hydrogen peroxide as a stressor with an exposure time of one hour. They found mouse astrocytes pretreated with higher concentrations of equilenin (100 nM, 1 μM, and 10 μM) had no significant difference between stressed and unstressed cultures. Their results indicate the higher concentrations of estrogens had greater effects on the cells than the stressor used. Their research also showed decrease cell viability at higher concentrations compared to controls for equilenin alone and the combination of equilin with equilenin. The current research partially supports these results. Higher concentrations with equilenin present had significantly lower cell viability. This further illustrates the results gathered from the trials using epinephrine as a stressor. Thus, higher concentrations of CEEs are neurologically harmful. To fully examine the neurological effects of CEEs, a cell line, such as primary astrocytes or neurons, should be examined in the future.

Hypoxia

A hypoxic environment was used to stress cells, a condition that mimicked a stroke *in vitro*. The current research found no significant difference across all estrogen treatments, indicating the estrogen treatments, alone or in combination, did not protect astrocytes. Previous research using different rat neurons found 17β-estradiol offered significant neuronal protection

compared to cells not treated with estradiol (Yousuf et al. 2014). These differences may be due to different cell lines. The current research used human astrocytes from a tumor whereas Yousuf et al. (2014) used rat neurons. The current research suggests estrogens are neither harmful nor helpful in protecting against a hypoxic environment. For future research, an increase in exposure time to a hypoxic condition may be necessary to see a greater difference across treatments including a decrease in viability. Additionally, measuring oxidative stress using flow cytometry would help better quantify the level of stress astrocytes undergo after being exposed to a hypoxic environment rather than only measure cell viability. Limitations such as directly measuring oxygen in the incubator may contribute to results gathered in the current study. Another constraint that exist in the current study was the limited exposure time of anoxic conditions. Thus, it is possible one hour exposure time was not enough to decrease cell viability where a significance would be observed. Thus, in the future, a longer exposure time of anoxic conditions should be examined. Additionally, the use of a gas chamber in the future would help measure the amount of oxygen directly.

The Women's Health Initiative study reported a positive relationship between ischemic stroke incidences and patients consuming CEEs. This study suggested CEEs were dangerous to take and detrimental to glial cells as well as possibly the individual. However, Zhao and Brinton (2006) found CEEs, specifically a combination of CEEs, to provide neuroprotection. Similarly, Grimes and Hughes (2015) reported CEEs to show an overall protective effect when astrocytes had exposure to hydrogen peroxide. In contrast, the current study found no significant protection of the CEE, equilenin, and found higher concentrations to be detrimental to cells. The fact that equilenin was harmful compared to estradiol alone supports results from previous research *in vitro* and *in vivo*. Women lack enzymes to properly metabolize equine estrogens. Thus, when

taken, the CEEs stay longer in the body (Hendrix et al. 2006). This is another important fact to consider before taking CEEs.

Short-Term Estrogen Treatment

It is important to emphasize that all astrocytes had only one hour of estrogen pre-treatment before toxin exposure. Consequently, this was a short-term exposure study which indicates the cellular harm with the higher concentrations of equilenin and equilenin with estradiol was not through transcriptional changes. Such changes take a longer time due to different gene expression pathways needed to travel. Therefore, the estrogens could be acting through plasma membrane receptor signaling. Additionally, alternative pathway where estrogens bind to receptors on the surface of the cell referred to as transmembrane G protein-coupled receptors may take place and activate signaling pathways (Yu et al. 2017). Once the estrogen binds to the receptor, conformational changes take place which activates other signaling pathways in the cell. These receptors can elicit faster responses compared to gene expression. It is known that equilenin can bind to ERα and ERβ receptors like estradiol. However, there may be differences in binding affinities that contribute to varying results (Luo et al. 2017; Grimes and Hughes 2015). Studying the receptor binding would be beneficial and would require blocking receptors and binding assays where one could look directly at the mechanism and signaling taking place. While no significant cellular protection was seen in the current research, certain estrogens may offer protection against other health complications such as cardiovascular disease as previous research has suggested (Simpkins et al. 2004). This research is valuable for women who are considering taking CEEs and gives insights on effects of estrogens at a short-term level. Thus, future research on the long-term effect of estrogens would be helpful to represent women who consume HRT for a long time.

Other Estrogens

Since estrone is most abundant (approximately 49%) compared to other estrogens in Premarin, it would be helpful to gain further insights on the cellular protective effects of estrone alone and in combination with other estrogens. Although previous research has found estrone to provide protection to rat neurons, using primary human astrocytes or primary human neurons would help better understand the effects estrogens have on women taking HRT (Zhao and Brinton 2006; Gatson et al. 2012). If primary neurons were examined, the preparation of the neuronal cultures would need astrocytes in order to survive *in vitro*. Thus, researching the effects estrogens have on primary neurons would provide great insight in the neurological effects of the brain when an individual consumes HRT. Examining other CEEs, such as equilin, effects in menopausal women would also be beneficial. Additionally, a longer-term study of the effects of equilenin, equilin, estrone, estradiol, pregestins, and other estrogens and hormones would be helpful in elucidating the neurological effects they have in the brain since there has been conflicting research on these estrogens and hormones (Zhoa and Brinton 2006; Brunner et al. 2010; Mosquera et al. 2014). The differences in estrogen structures may contribute to the differences in results across various research using different estrogens.

Limitations

Although the current research gave insight to estrogen's effect on human astrocytes, limitations are present which includes the cell line. While the astrocytoma cell line has the morphology of astrocytes, questions about the molecular changes from primary astrocytes remain. The next step in this research would be to use primary human astrocytes. Despite the fact this was an *in vitro* study using an astrocytoma cell line, it supports other studies and is a reason

to do other *in vivo* studies on these estrogens to examine neurological effects. Time is a sensitive factor when taking estrogens to protect against Alzheimer's disease (Marriott and Wenk 2004).

Although no significant protection was found in estrogen treatments, this study still offers implications for estrogens, specifically estradiol, as safer to take at lower doses compared to a CEE or a combination of the two at higher concentrations. Future research on primary human astrocytes and primary human neurons would help clarify the effects of estrogens. Much research has found protection in rat neurons and astrocytes, but less research has been done on primary human neurons (Zhao and Brinton 2006; Grimes and Hughes 2015).

The current study initially used flow cytometry to measure the reactive oxidative species, cells under oxidative stress. The value of doing such assays allows better examination of cellular activity and effects of estrogens as well as stressor effects. Analysis of the results were not reported, however, due to system limitations when it came to optimizing the assay for oxidative stress. If this assay is optimized in the future, it could be beneficial to adding information to what is already known about certain estrogens protecting astrocytes as well as stressor effect. Additionally, it would be helpful in confirming the concentrations of the stressors used affected oxidative stress measurements.

Future research

In the future, using a higher concentration of the stressors where lower viability will be measurable would help examine estrogen effects. Examination of how different sex cells (male vs. female) from nerve tissue respond to the estrogen treatments would also help gain further insight on estrogen's effects since previous research found the protective effects of estrogens to be sex specific. Since astrocytes are closely associated with the brain endothelial barrier, it would

be beneficial in examining the protective effects estrogens may have on neurons and other cell lines of astrocytes, such as primary astrocytes. The human astrocytes (1321N1 from Sigma-Aldrich) did not state if they were from a male or female. Since Premarin is composed of nearly fifty percent estrone, future research on estrone protective effects on astrocytes would be beneficial since other research found estrone provided significant protection to neurons in the parietal cortex and hippocampus (Johnsen and Murphy 2010). Testing the affinity of binding receptors for each estrogen and using an estrogen receptor blocker to examine the effects of estrogen binding with estradiol and equilenin would help understand the different results gathered from each estrogen. The information gathered from this research helps further understand the harmful effects CEEs may have on the body when taken HRT. Questions remain concerning the effects estradiol and CEEs have protecting against strokes and other health complications *in vivo*.

Table 1. Results from comparing means using a two-way ANOVA for astrocytes pretreated with estradiol, equilenin, and estradiol with equilenin for one hour followed by 100 μM epinephrine exposure for one hour. Log-transformed data are italicized. P-values < 0.05 indicate significance.

Type of stressor	**Type of estrogen**	**Source of Variation**	**d.f.**	**F**	**P (p<0.05)***
Epinephrine	Estradiol	Stressor present	*1*	*0.53*	*0.47*
		Estrogen conc.	*4*	*3.02*	*0.02**
		Stressor X estrogen conc.	*4*	*0.09*	*0.98*
	Equilenin	Stressor present	1	0.33	0.57
		Estrogen conc.	4	13.89	< 0.001*
		Stressor X estrogen conc.	4	0.7	0.6
	Estradiol+Equilenin	Stressor present	1	0.02	0.89
		Estrogen conc.	4	7.66	<0.0001*
		Stressor X estrogen conc.	4	0.41	0.8

Table 2. Two-way ANOVA results for astrocytes pretreated with estradiol, equilenin, and estradiol with equilenin for one hour followed by 1μM cortisol exposure for one hour. Log-transformed data are italicized. P-values < 0.05 indicate significance.

Type of stressor	Type of estrogen	Source of Variation	d.f.	F	P (p<0.05)*
Cortisol	Estradiol	Stressor present	*1*	*1.53*	*0.22*
		Estrogen conc.	*4*	*1.29*	*0.28*
		Stressor X estrogen conc.	*4*	*0.74*	*0.57*
	Equilenin	Stressor present	*1*	*0.08*	*0.78*
		Estrogen conc.	*4*	*4.01*	*0.005**
		Stressor X estrogen conc.	*4*	*1.37*	*0.25*
	Estradiol+Equilenin	Stressor present	*1*	*0.17*	*0.68*
		Estrogen conc.	*4*	*9.89*	<0.0001*
		Stressor X estrogen conc.	*4*	*0.61*	*0.65*

Table 3. One-way ANOVA results for astrocytes pretreated with estradiol, equilenin, and estradiol with equilenin for one hour followed low oxygen exposure for one hour. P-values <0.05 indicate significance.

Type of stressor	Type of estrogen	Source of Variation	d.f.	F	P
Oxygen	Estradiol	Estrogen conc.	5	1.61	0.178
	Equilenin	Estrogen conc.	5	1.83	0.127
	Estradiol+Equilenin	Estrogen conc.	5	1.9	0.112

Table 4. Mean and standard deviation of astrocytes treated with estradiol and stressed with epinephrine.

	Epinephrine		Estradiol		
	Control	10 nM	100 nM	1 μM	10 μM
Mean	0.2	0.66	0.91	1.13	0.74
Standard Error	019	0.21	0.21	0.21	0.19

Table 5. Mean and standard deviation of astrocytes treated with equilenin and stressed with epinephrine.

	Epinephrine	Equilenin			
	Control	10 nM	100 nM	1 μM	10 μM
Mean	0.92	0.68	0.66	0.66	0.164
Standard error	0.07	0.008	0.08	0.08	0.07

Table 6. Mean and standard deviation of astrocytes treated with a combination of estradiol and equilenin and stressed with epinephrine.

	Epinephrine		Estradiol and Equilenin		
	Control	10 nM	100 nM	1 μM	10 μM
Mean	0.92	0.6	0.63	0.51	0.26
Standard error	0.08	0.09	0.09	0.09	0.09

Table 7. Mean and standard deviation of astrocytes treated with estradiol and stressed with cortisol.

	Cortisol		Estradiol		
	Control	10 nM	100 nM	1 μM	10 μM
Mean	0.71	0.74	0.82	0.53	0.78
Standard error	0.09	0.11	0.11	0.11	0.1

Table 8. Mean and standard deviation of astrocytes treated with equilenin and stressed with cortisol.

	Cortisol		Equilenin		
	Control	10 nM	100 nM	1 μM	10 μM
Mean	0.71	0.68	0.77	0.67	0.45
Standard error	0.09	0.11	0.11	0.1	0.01

Table 9. Mean and standard deviation of astrocytes treated with combined estrogens and stressed with cortisol.

	Cortisol		Estradiol	+ equilenin	
	Control	10 nM	100 nM	1 μM	10 μM
Mean	0.71	0.72	0.51	0.66	0.23
Standard error	0.08	0.1	0.1	0.09	0.09

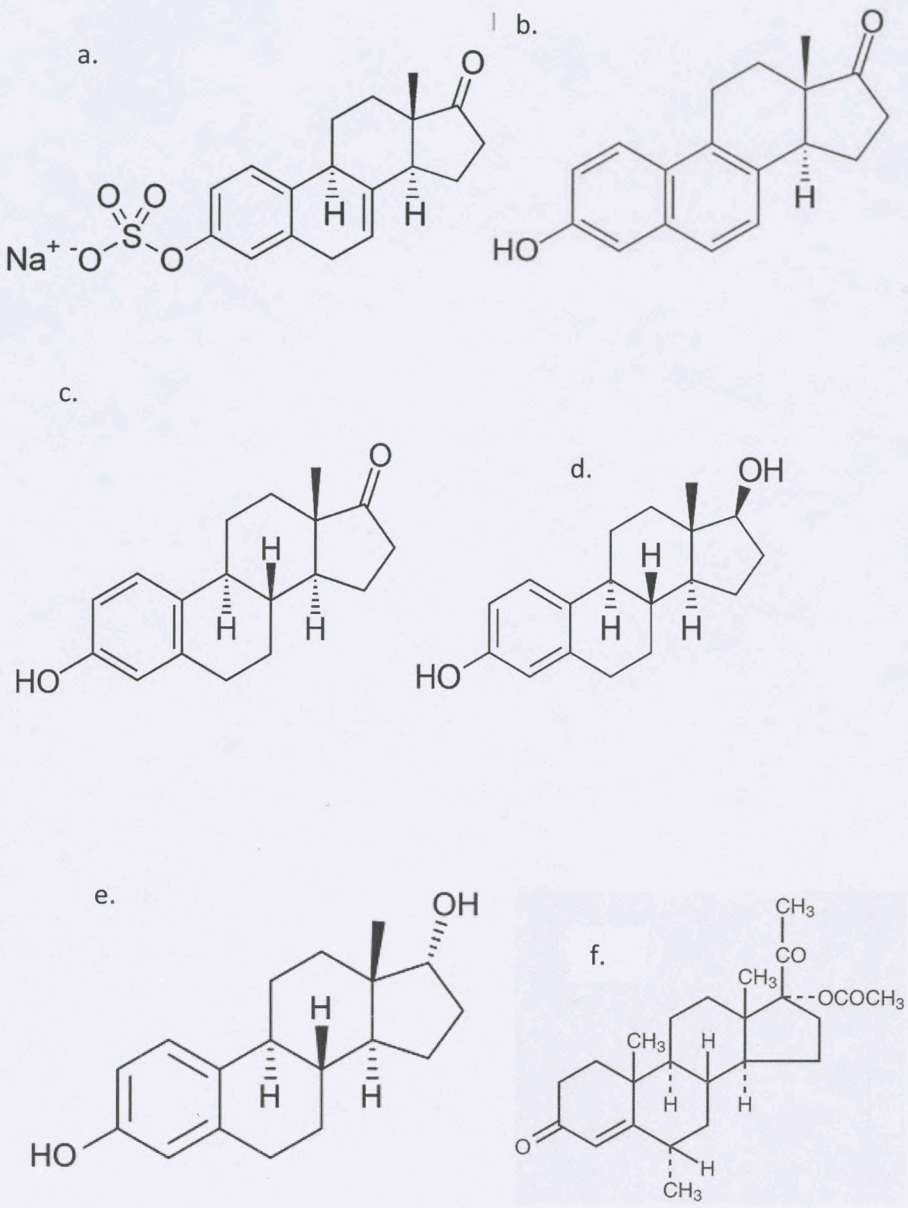


Fig. 1. Chemical formula of a.) Premarin, b.) Equilenin, c.) Estrone d.) 17β-estradiol, e.) 17α-estradiol, and f.) Prempro Adapted from National Center for Biotechnology Information. PubChem Compound Database retrieved from https://pubchem.ncbi.nlm.nih.gov/compound (accessed Nov., 5, 2017)

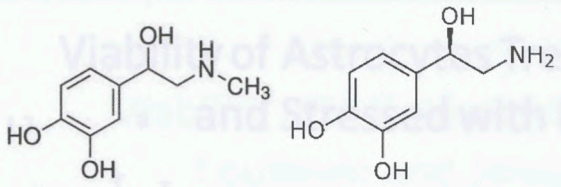


Figure 2. Chemical structure of epinephrine (left) and norepinephrine (right). Adapted from National Center for Biotechnology Information. PubChem Compound Database retrieved from https://pubchem.ncbi.nlm.nih.gov/compound (accessed Oct., 1, 2017).

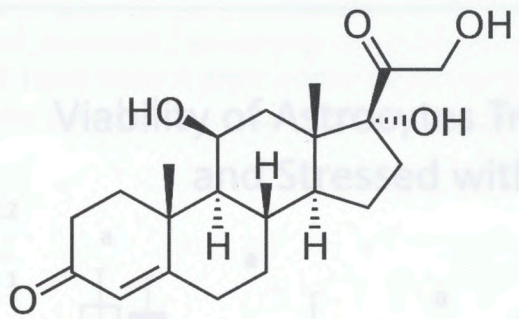


Figure 3. Chemical formula of cortisol. Adapted from National Center for Biotechnology Information. PubChem Compound Database retrieved from https://pubchem.ncbi.nlm.nih.gov/compound/hydrocortisone (accessed Oct., 1, 2017).

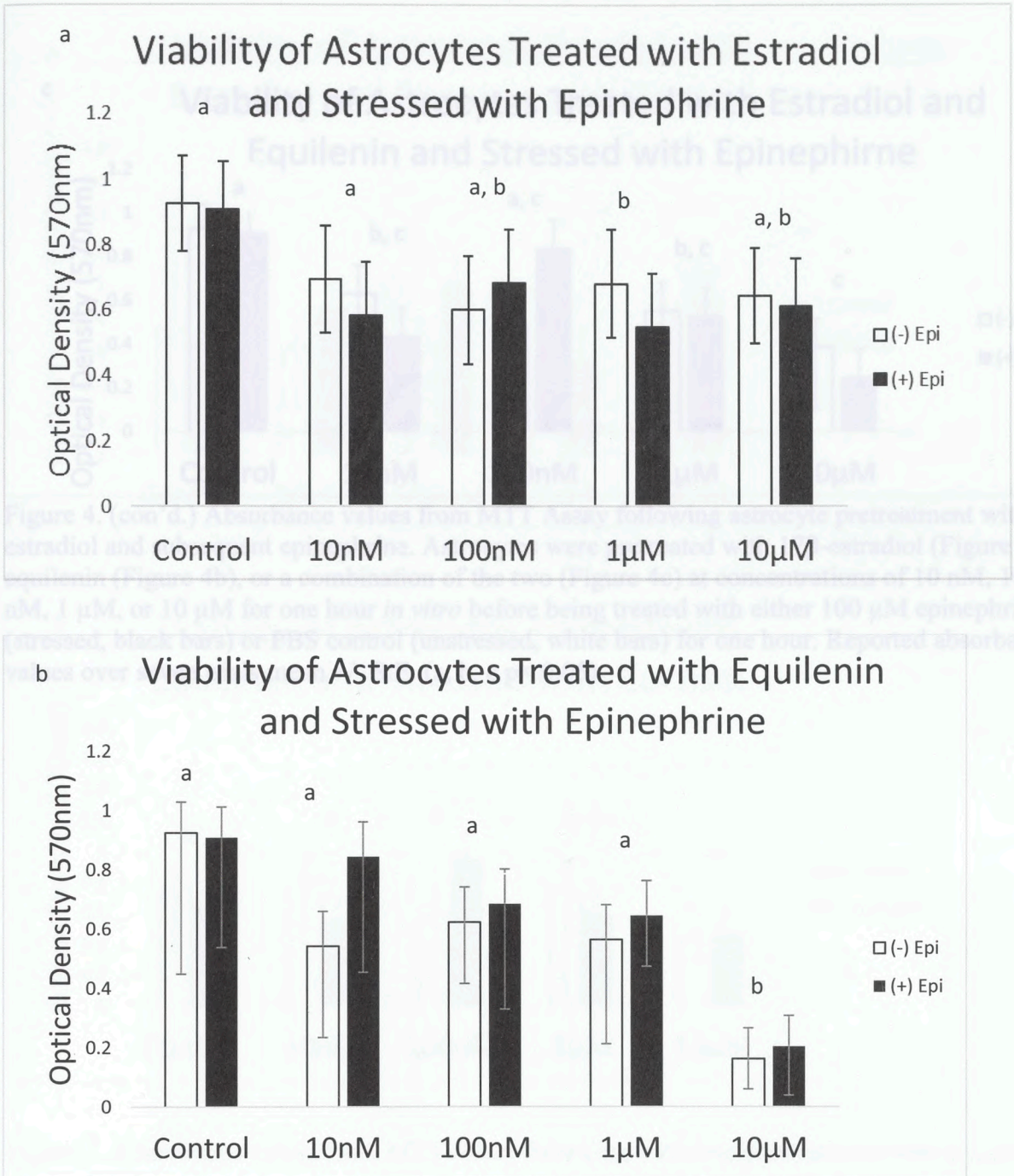


Figure 4. Absorbance values from MTT Assay following astrocyte pretreatment with estradiol and subsequent epinephrine. Astrocytes were pretreated with 17β-estradiol (Figure 4a), equilenin (Figure 4b), or a combination of the two (Figure 4c) at concentrations of 10 nM, 100 nM, 1 µM, or 10 µM for one hour *in vitro* before being treated with either 100 µM epinephrine (stressed, black bars) or PBS control (unstressed, white bars) for one hour. Reported absorbance values over seven trials mean +/- S.E. (a, b, c $p < 0.05$).

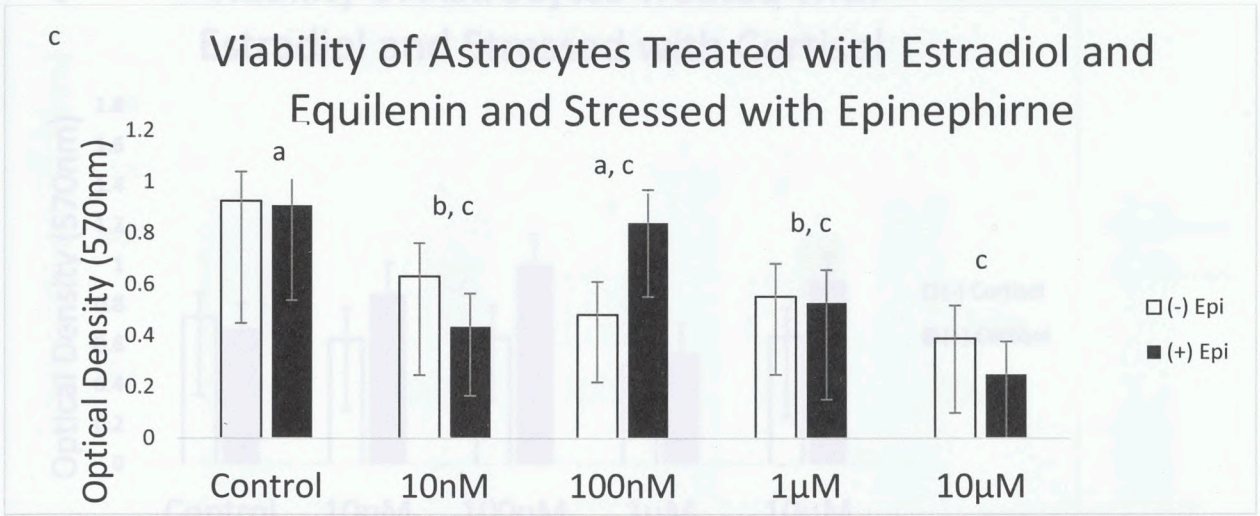


Figure 4. (con'd.) Absorbance values from MTT Assay following astrocyte pretreatment with estradiol and subsequent epinephrine. Astrocytes were pretreated with 17β-estradiol (Figure 4a), equilenin (Figure 4b), or a combination of the two (Figure 4c) at concentrations of 10 nM, 100 nM, 1 μM, or 10 μM for one hour *in vitro* before being treated with either 100 μM epinephrine (stressed, black bars) or PBS control (unstressed, white bars) for one hour. Reported absorbance values over seven trials mean +/- S.E. (a, b, c $p < 0.05$).

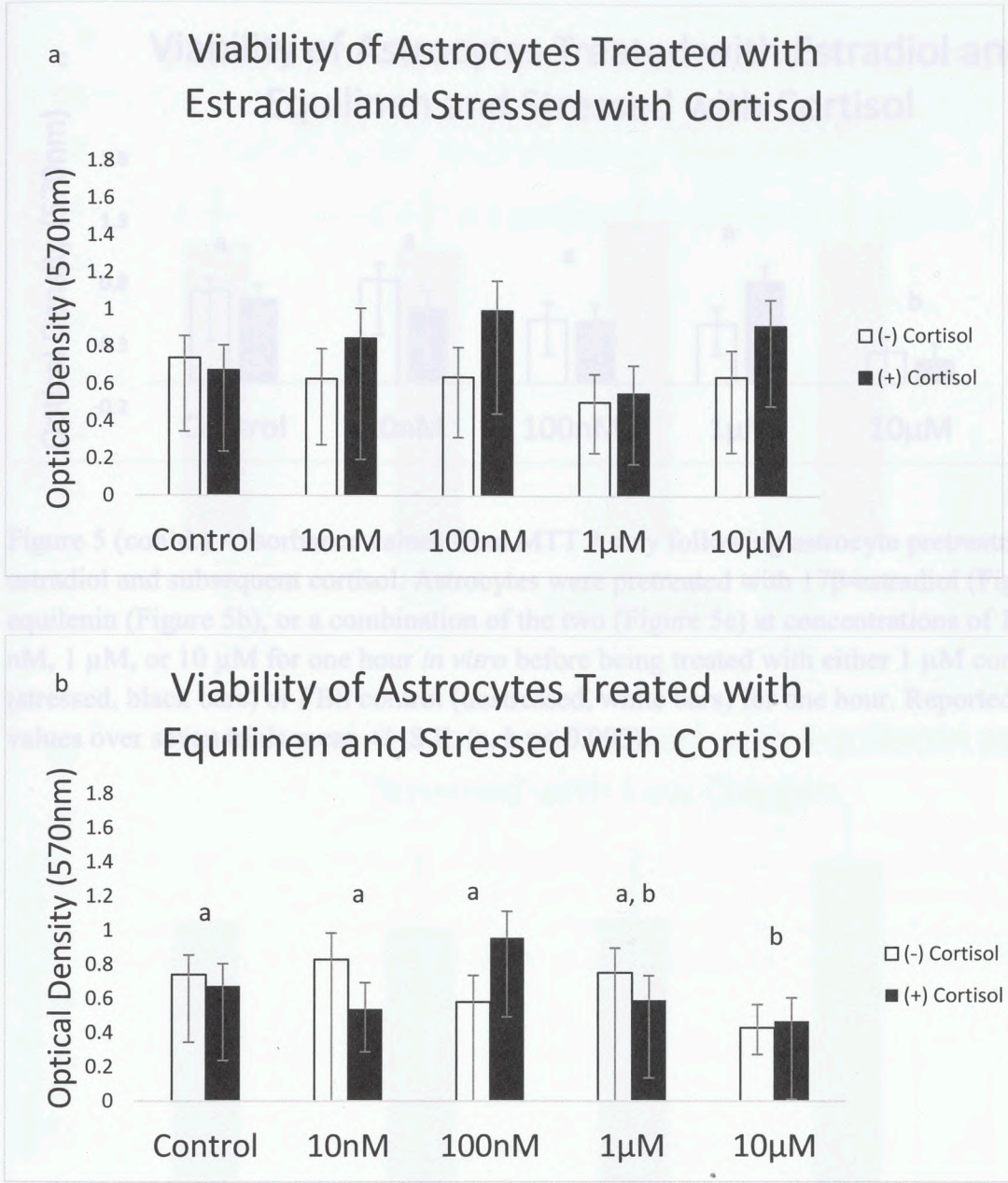


Figure 5. Absorbance values from MTT Assay following astrocyte pretreatment with estradiol and subsequent cortisol. Astrocytes were pretreated with 17β-estradiol (Figure 5a), equilenin (Figure 5b), or a combination of the two (Figure 5c) at concentrations of 10 nM, 100 nM, 1 μM, or 10 μM for one hour *in vitro* before being treated with either 1 μM cortisol (stressed, black bars) or PBS control (unstressed, white bars) for one hour. Reported absorbance values over seven trials mean +/- S.E. (a, b $p < 0.005$).

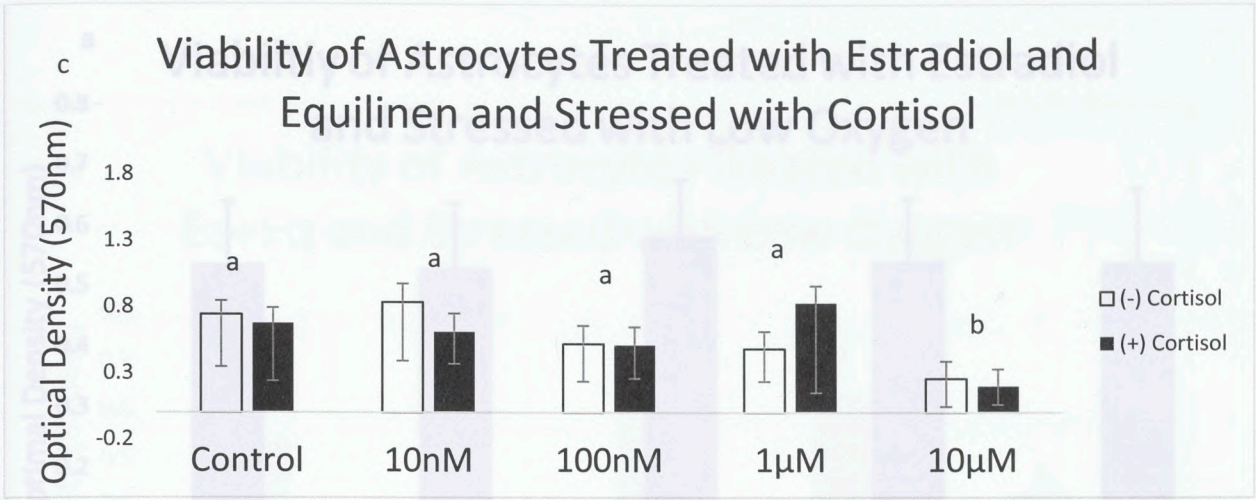
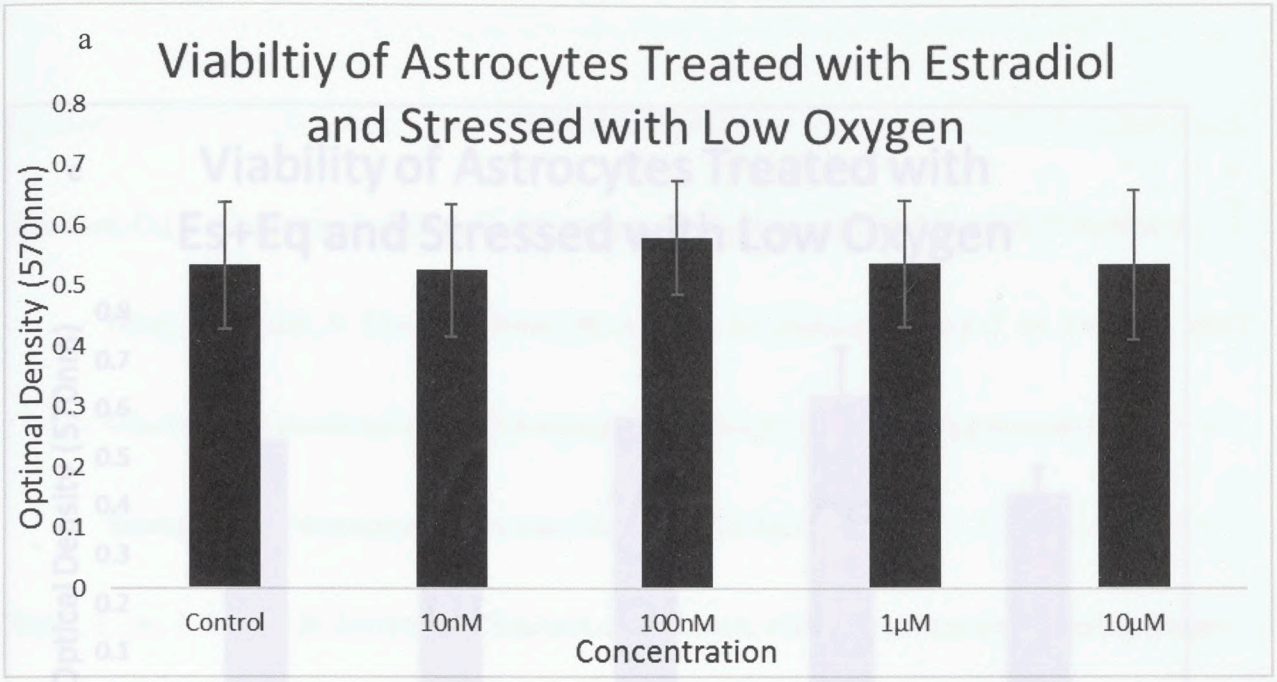


Figure 5 (con'd.) Absorbance values from MTT Assay following astrocyte pretreatment with estradiol and subsequent cortisol. Astrocytes were pretreated with 17β-estradiol (Figure 5a), equilenin (Figure 5b), or a combination of the two (Figure 5c) at concentrations of 10 nM, 100 nM, 1 μM, or 10 μM for one hour *in vitro* before being treated with either 1 μM cortisol (stressed, black bars) or PBS control (unstressed, white bars) for one hour. Reported absorbance values over seven trials mean +/- S.E. (a, b $p < 0.005$).



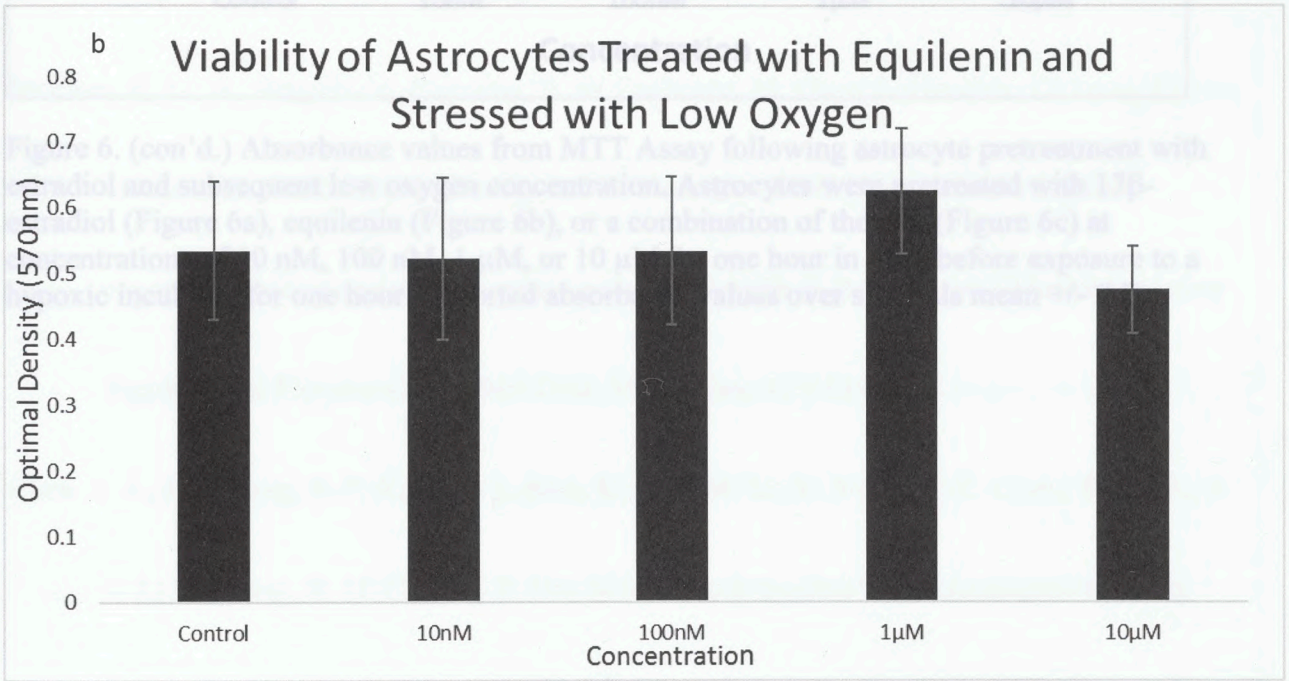


Figure 6. Absorbance values from MTT Assay following astrocyte pretreatment with estradiol and subsequent low oxygen concentration. Astrocytes were pretreated with 17β-estradiol (Figure 6a), equilenin (Figure 6b), or a combination of the two (Figure 6c) at concentrations of 10 nM, 100 nM, 1 µM, or 10 µM for one hour in vitro before exposure to a hypoxic incubator for one hour. Reported absorbance values over six trials mean +/- S.E.

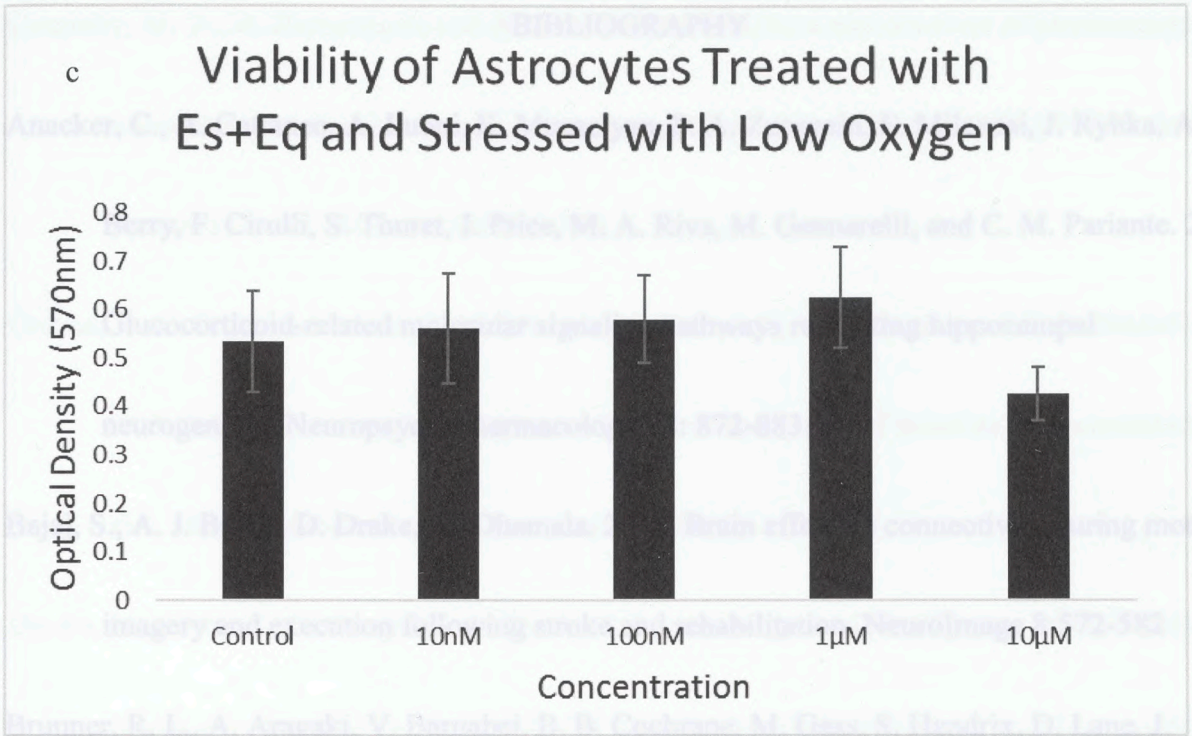


Figure 6. (con'd.) Absorbance values from MTT Assay following astrocyte pretreatment with estradiol and subsequent low oxygen concentration. Astrocytes were pretreated with 17β-estradiol (Figure 6a), equilenin (Figure 6b), or a combination of the two (Figure 6c) at concentrations of 10 nM, 100 nM, 1 μM, or 10 μM for one hour in vitro before exposure to a hypoxic incubator for one hour. Reported absorbance values over six trials mean +/- S.E.

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